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UPTAKE AND METABOLISM OF FREE COBALAMIN BY CULTURED HUMAN FIBROBLASTS

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ABSTRACT

This study was undertaken to investigate the uptake and metabolism of free cobalamin (vitamin B_{12}) by cultured human skin fibroblasts. It was shown that normal diploid fibroblasts in culture take up free cobalamin (Cbl) via a saturable, mediated process which is inhibited by sulfhydryl reagents and inhibitors of protein synthesis but which is relatively unaffected by inhibitors of glycolysis and respiration. Cobalamin taken up in this manner becomes associated with intracellular Cbl-dependent apoenzymes. A similar process was shown to occur in skin fibroblasts from patients with a deficiency of transcobalamin II (TC II), the plasma protein which mediates the normal process of Cbl uptake. It was observed that normal skin fibroblasts in culture synthesize TC II and secrete it into incubation medium. Synthesis of the transport protein does not occur in fibroblasts from TC II-deficient patients, indicating that the observed uptake process for free Cbl is not mediated by TC II. A mechanism by which an uptake process for free Cbl might serve a function in normal Cbl metabolism is proposed.

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INTRODUCTION

The elucidation of pathways of intermediary metabolism has often been closely linked to the study of clinical phenomena arising from circumscribed lesions, congenital or acquired, which disrupt the normal sequence of metabolic events. Perhaps no system better portrays this interplay of clinical observation and biochemical investigation than the study of the metabolism of cobalamin (vitamin B_{12}). The observation in 1926 that liver extracts could be used to treat megaloblastic anemia (1) led to the further understanding of the role of vitamin B_{12} and gastric intrinsic factor deficiency in the pathophysiology of pernicious anemia and culminated in the isolation of the vitamin in 1948 (2). Since that time, inborn errors have been observed involving almost every aspect of cobalamin metabolism and their study has fostered the evolving understanding of the normal metabolic processes involved in the molecule's absorption, transport, and conversion to active coenzymes.

This study was designed to explore further one aspect of cobalamin metabolism and it too has its roots in the investigation of the pathophysiology of an inborn metabolic defect. It has been observed that patients with a congenital deficiency of the transport protein (transcobalamin II) responsible for the cellular uptake of cobalamin, who have the symptoms of severe cobalamin deficiency, respond to pharmacologic doses of vitamin B_{12}, thus suggesting that cells are capable of taking up and utilizing free as well as protein-bound cobalamin. These observations prompted this *in vitro* investigation of the uptake and metabolism of free cobalamin by human fibroblasts with the goal of further illuminating the normal processes of cobalamin metabolism while explaining the observed pathologic phenomena in the clinical model.

An understanding of current concepts of cobalamin metabolism depends on a grasp of the salient features of the vitamin's structure and function. The remainder of this introduction will survey relevant aspects of the chemistry of the cobalamin compounds, their metabolism, and their function as intracellular cofactors, and includes a brief discussion of the clinical features of cobalamin-related metabolic disease.

Chemical Structure of Cobalamin

The complex three-dimensional structure of cobalamin (vitamin B_{12}) was elucidated using x-ray crystallography in 1956 by Hodgkin et al. (3). The molecule (Fig. 1) consists of a planar corrin ring around a central cobalt atom, with a side chain extending below the plane of the ring. The side chain contains a 5,6-dimethylbenzimidazole ring with ribose and phosphate moieties, and attaches via a Co-N coordination linkage to the benzimidazole ring. The phosphate moiety provides a second link to the corrin ring. The different cobalamin compounds are characterized by the radicals in covalent linkage to the cobalt nucleus above the plane of the corrin ring. Three such compounds have been found in mammals: hydroxocobalamin, methylcobalamin, and adenosylcobalamin. The commonly isolated form, cyanocobalamin, is not found *in vivo* and is an artifact of isolation.

The central cobalt atom of the cobalamin molecule can exist in a monovalent, divalent, or trivalent state. Hydroxocobalamin, the naturally occurring form of the vitamin, has a trivalent cobalt atom; in its metabolism to the active coenzyme compounds, adenosylcobalamin and methylcobalamin, the cobalt must be reduced to a monovalent state. This sequential reduction from Co^{+++} to Co^{+} in the formation of

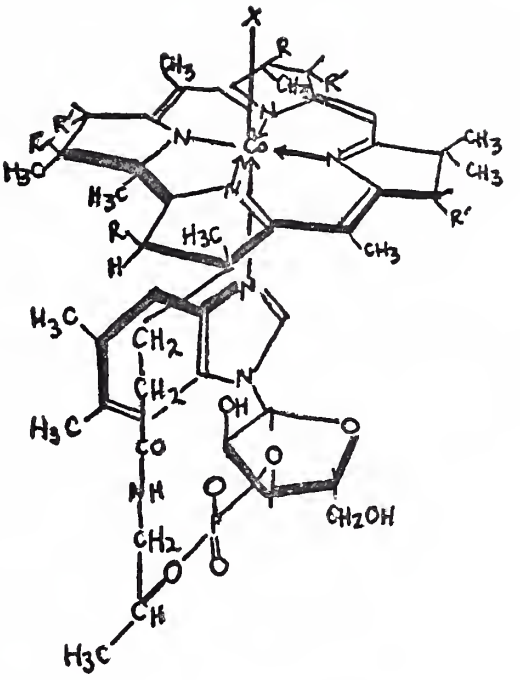


FIGURE 1. Structure of the cobalamin molecule. R = CH_2CONH_2; R' = $CH_2CH_2CONH_2$; X = -OH,-CN,-CH_3, or 5'-deoxy-5'-adenosyl.

adenosylcobalamin has been shown in bacteria to be mediated by specific enzymes (4); this process, and the evidence for similar cobalamin reductases in mammalian cells, will be discussed in connection with the metabolism of adenosylcobalamin.

Adenosylcobalamin and Methylmalonate Metabolism

Adenosylcobalamin (AdoCbl) was the first cobalamin coenzyme to be described and partially purified. In 1958, Barker et al. (5) described a coenzyme form of "pseudovitamin B_{12}" required for the isomerization of glutamate to β-methylaspartate in Clostridium tetanomorphum. Isolation yielded a substance which, on exposure to light, released adenine and a substance with an absorption spectrum similar to cobalamin. The coenzyme, adenosylcobalamin, has 5'-deoxy-5'-adenosyl in the sixth coordination position of the cobalamin molecule. Adenosylcobalamin and methylcobalamin are unique in that they are the only naturally occurring substances known to have a direct carbon-cobalt bond.

A role for AdoCbl as a coenzyme in mammals--specifically, for the conversion of methylmalonyl CoA to succinyl CoA--was proposed in 1958 by Smith and Monty (6) and subsequently confirmed (7,8). Gurnani et al. (7) reported that mitochondrial preparations of livers from cobalamin-deficient rats showed normal rates of methylmalonate formation but markedly reduced rates of succinate production; addition of "vitamin B_{12} cofactor" (AdoCbl) restored this activity in vitro. Stern and Friedman (8) reported similar results in ox liver fractions.

Methylmalonyl CoA mutase, the enzyme for which AdoCbl is a coenzyme, converts methylmalonyl CoA to succinyl CoA. Methylmalonyl CoA is an intermediate in the pathway of propionate metabolism, as well as a

product of thymine metabolism. Thymine is only a minor source of methylmalonyl CoA; the main contribution to the intracellular production of methylmalonyl CoA is from propionyl CoA, an intermediate in the final common pathway for the catabolism of branched chain amino acids, cholesterol, and odd chain fatty acids (9) (see Fig. 2).

The metabolism of OH-Cbl to AdoCbl was first studied in bacteria. Walter et al. described specific reductases responsible for the sequential reduction of cob[III]alamin to cob[I]alamin in cell-free extracts of C. tetanomorphum (4); they reported two NADH-dependent flavoproteins distinguishable on the basis of their cofactor requirements and thermolability. Subsequently, they reported isolation and purification of a Cbl-specific adenosyltransferase (10). Hence, evidence favors a three-step metabolic pathway for the production of AdoCbl in bacteria: two sequential reductions of OH-Cbl followed by adenosylation of cob[I[alamin; each step requires a specific enzyme.

In higher animals, AdoCbl synthesis has been shown to be a mitochondrial process: Mahoney et al. (11) reported AdoCbl synthetic capability in mitochondria-enriched subcellular fractions of fibroblasts in a system which bypassed the first reduction step; subsequently, Fenton and Rosenberg (12) have shown that intact, purified rat liver mitochondria can convert OH-Cbl to AdoCbl without bypassing any part of the pathway. Despite these studies, efforts to confirm an enzymatic pathway similar to that in bacteria have been unsuccessful to date. However, indirect evidence that the pathway for the synthesis of AdoCbl is enzymatic is provided by the observation of inborn errors of metabolism which result in an inability to convert OH-Cbl to AdoCbl (13).

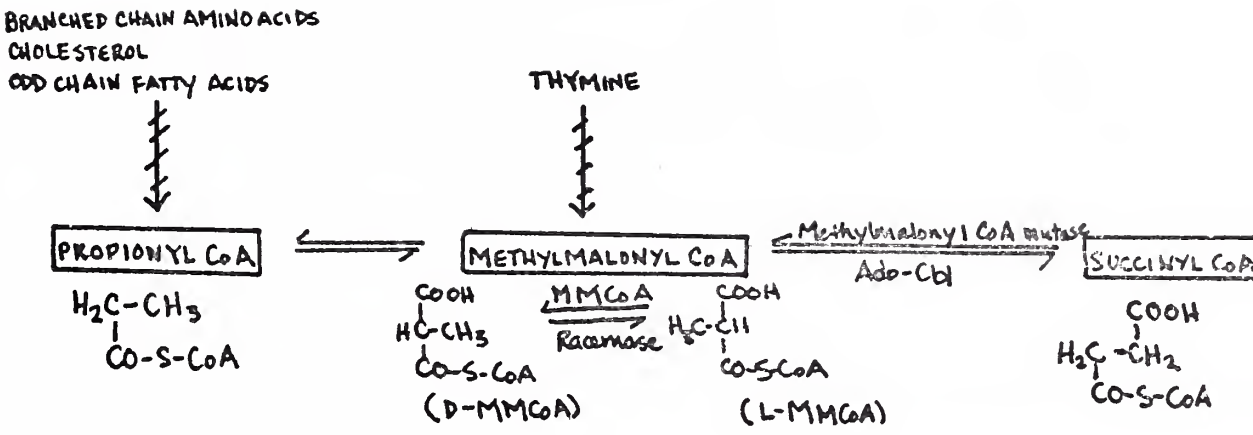


FIGURE 2. Role of AdoCbl in the pathways of propionate and methylmalonate metabolism. Cross-hatched arrows indicate multiple reactions.

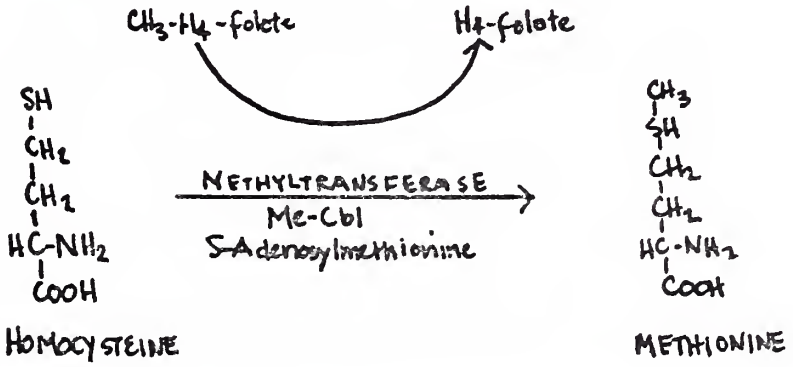


FIGURE 3. Conversion reaction for homocysteine to methionine for which MeCbl is a cofactor.

Methylcobalamin and Homocysteine Metabolism

In 1956, Helleiner and Woods (14) reported the facilitation of methionine synthesis by adding Cbl to cell-free extracts of E. coli. Ten years later, Weissbach and Taylor (15) reported that methylcobalamin (MeCbl) is a cofactor in the remethylation of homocysteine to methionine; they isolated a cobamide-enzyme complex with transmethylase activity from E. coli which, in the presence of N^5-methyltetrahydrofolate and S-adenosylmethionine, could generate methionine from homocysteine (16) (see Fig. 3). Using ^{14}C-labelled methyltetrahydrofolate, they demonstrated a ^{14}C-methylcobalamin-enzyme intermediate and proposed a mechanism by which the methyl group is transferred first from methyltetrahydrofolate to the Cbl-enzyme complex, and subsequently to homocysteine. The transfer of the methyl group to the Cbl-enzyme complex is facilitated by the reduction of the Co^{+++} to Co^{+}, which generates a strong nucleophile (15,16).

The significance of this cobalamin dependent reaction is twofold: in addition to the metabolism of homocysteine to methionine, the methyltransferase reaction serves as a source for the regeneration of demethylated tetrahydrofolate. Through a mechanism which is still not clearly established, this link to the pathway of folate metabolism plays an important role in the clinical manifestations of disorders of cobalamin metabolism (9). This will be discussed further with those disorders.

Cobalamin Metabolism

The synthesis of cobalamin (Cbl) is restricted to microorganisms; consequently, higher animals must derive Cbl from dietary sources or from their intestinal flora. The mammalian mechanism of transport, uptake,

and metabolism of Cbl is an elaborate one which carefully conserves and maximally utilizes the trace quantities of the vitamin present in the external environment. The absorption of dietary Cbl is facilitated by gastric intrinsic factor (IF), a glycoprotein secreted by the gastric mucosa which competes for the Cbl in ingested animal protein and binds the vitamin in the intestinal lumen (17). The successful trapping of Cbl by IF appears to depend on partial acid digestion of the ingested protein, because some achlohydric and post-partial gastrectomy patients malabsorp protein-bound Cbl despite normal absorption of crystalline Cbl (18).

The IF-Cbl complex then binds to ileal receptors via a rapid, Ca^{++}-dependent process which is unaffected by temperature or metabolic inhibitors (19,20). The receptors are specific for the IF-Cbl complex; binding is unaffected by the presence of large excesses of free Cbl, free IF, or other Cbl-binders found in plasma, saliva, granulocytes, and breast milk (21). However, the affinity of the complex for the receptor is apparently unchanged by binding of other Cbl compounds to IF; if a Cbl compound can effectively bind to IF, the complex can bind to the intestinal wall (22).

Once bound to the ileal receptors, Cbl is absorbed by a poorly-understood mechanism by which the vitamin is taken into and transported across the intestinal epithelial cell. It is not clear whether or not IF is taken up with the Cbl, and little is known about the intracellular transport of the vitamin, but it is ultimately released free into the portal blood (23,24). This phase of absorption is slow; although the IF-Cbl complex associates with ileal receptors within minutes, peak plasma levels of Cbl are not achieved until about eight hours after ingestion (25).

The free Cbl is immediately bound by plasma globulins as it enters the bloodstream. Almost all newly absorbed Cbl binds to transcobalamin II (TC II), a β-globulin isolated in 1963 by Hall and Finkler (26). TC II is synthesized by the liver and possibly by other organs (27,28) and represents 80-100% of unsaturated Cbl-binding capacity in human plasma (23). Cobalamin bound to TC II has a rapid turnover rate and most disappears from plasma within a few hours (29). The remainder of newly-absorbed Cbl binds to transcobalamin I (TC I), an α-globulin thought to be derived from granulocytes (30). TC I is one of the "R binder" proteins--a series of Cbl binders with identical amino acid sequences, which can be distinguished by their associated carbohydrate moieties, and which are secreted by a number of tissues including granulocytes, saliva, and breast milk (31,32). The functional significances of these secretory binding proteins is unclear (23). The TC I-bound Cbl turns over very slowly and when newly absorbed vitamin comes into equilibrium with total body Cbl, TC I-bound Cbl represents 75 percent of Cbl in plasma (33).

When Hall and Finkler first reported the isolation of TC II in 1963, they suggested that it might prove important in the movement of Cbl from the gut to other tissues (26). Since that time, the TC II-Cbl complex has been studied extensively both in vivo and in vitro. TC II has been shown to facilitate the uptake of Cbl by HeLa cells (34), erythrocytes (35), Ehrlich ascites tumor cells (36), L1210 leukemia cells (37), and cultured human fibroblasts (38).

Like the absorption of the IF-Cbl complex, TC II-Cbl uptake has been shown to be a biphasic process. Parenchych and Cooper (36,39) showed that TC II-Cbl uptake by Ehrlich ascites cells and by HeLa cells

consists of a rapid, Ca^{++}-dependent, temperature- and energy-independent phase followed by a slower, temperature-dependent process which can be inhibited metabolically. They suggested that the first component represents binding to a membrane receptor--a passive, physico-chemical reaction, and that the second component corresponds to internalization--an active transport process. Youngdahl-Turner and colleagues (40) studied the kinetics of these two components separately in cultured human fibroblasts, and provided strong evidence of this interpretation. Studies at 4° showed saturable, Ca^{++}-dependent binding specific for the TC II moiety of the complex. More than 95 percent of the Cbl bound could be released by trypsin, indicating that Cbl is not internalized at 4°; internalization occurred at 37° by a process which was pH-dependent and inhibited by metabolic inhibitors of endocytosis (40,41). Studies with double-labelled ^{125}I-TC II-CN-[^{57}Co]Cbl showed that both labels were taken up simultaneously, suggesting that the TC II-Cbl complex is taken up intact (41).

Pletsch and Coffey (42) studied the mechanism of uptake of the TC II-Cbl complex by cell fractionation studies of rat liver after intravenous injection of Cbl, and reported a transient association of Cbl with lysosomal fractions preceding its appearance in large quantities in the mitochondrial and soluble fractions. Further evidence for the association with lysosomes is provided by the observation that chloroquine, an inhibitor of lysosomal activity, prevents the release of Cbl from TC II-Cbl complex and consequently inhibits the synthesis of coenzymes (41).

Thus, evidence has accumulated to suggest the following sequence of events: 1) the TC II-Cbl complex binds to specific receptors on the

cell surface; 2) the complex is taken up intact via adsorptive endocytosis; 3) the endocytotic vesicle fuses with a lysosome; and 4) the TC II-Cbl complex is degraded, allowing release of free Cbl into the cytosol with subsequent conversion to active coenzymes (41,42). The mechanism by which free Cbl is transported out of the lysosome and into mitochondria is unknown.

Clinical Features of Cobalamin Related Disorders

The clinical manifestations of Cbl-related disorders are pleomorphic. The clinical features of vitamin deficiency usually differ widely from the findings associated with inborn errors affecting intracellular coenzyme activity; it is this dissociation which suggests that Cbl may well serve another as yet undetermined function in mammals. I will first discuss the cardinal manifestations of Cbl deficiency--megaloblastic anemia and neurologic degeneration, and the primary presenting signs of disorders of intracellular Cbl metabolism--methylmalonic acidemia (-uria), homocystinemia (-uria), and ketoacidosis; I will then describe the manifestations of TC II deficiency and its implications with regard to the metabolic consequences of this inborn error.

Megaloblastic Anemia -- Megaloblastic changes involve all formed elements of blood and are usually associated with either Cbl or folate deficiency, though they may occur as a result of other metabolic disorders which impair DNA synthesis (43). Megaloblastic anemia is characterized by a normochromic, macrocytic anemia, leukopenia with hypersegmented polymorphonuclear leukocytes, and thrombocytopenia. The pancytopenia is primarily a result of ineffective hematopoiesis (43-45); in the erythroid series, peripheral hemolysis also plays a role. The bone marrow is normocellular

or hypercellular and shows megaloblastic transformation of all cell lines. The hallmark of the megaloblastic process is "nuclear-cytoplasmic asynchronism" (43)--the development of cells with mature cytoplasmic elements and an immature nucleus. Erythrocytes are large with mature cytoplasm--appearing either basophilic or containing hemoglobin, with fine granular nuclei characteristic of early phases of erythroid maturation. Leukocytes and megakaryocytes are less dramatically changed in appearance, but also show signs of nuclear-cytoplasmic asynchronism, as well as increased marrow destruction (44,45).

The pathophysiologic basis for megaloblastosis is thought to be impaired DNA synthesis with resultant unbalanced growth, maturation arrest, and cell death. The primary lesion is probably a defect in the synthesis of DNA precursors, notably thymidine (46), resulting in impairment of DNA synthesis in the absence of a block in RNA synthesis. Megaloblastic cells have greatly increased amounts of RNA with normal levels of DNA (47); DNA synthesis can be increased <u>in vitro</u> by the addition of precursors (48,49). Studies of bacterial systems with isolated impaired ability to make deoxyribonucleosides have shown the consequences to be unbalanced growth, increased cell size, increased RNA content and very slow DNA synthesis. This leads to slow and abnormal proliferation, and rapidly decreasing viability (50). The same process appears to occur in Cbl- or folate-deficient bone marrow. Maturation of erythrocytes in megaloblastic anemia is delayed, there is disruption of the normal cell cycle, with a prolongation of the G1 (pre-DNA synthesis) phase and delay of the S (DNA-synthetic) phase (48,49,51). The cells enlarge, and are predisposed to abnormal maturation and/or early death. This process can be reversed by thymidine (52), folate, or Cbl.

The etiology of impaired DNA synthesis in megaloblastic anemia is not well established. The most intriguing clue lies in the link between Cbl and folate. Megaloblastic anemia occurs with deficiency of either folate or Cbl, and pharmacologic doses of one can partially or completely reverse the megaloblastosis induced by deficiency of the other. This interrelationship implies strongly that the basis of megaloblastic transformation lies in a Cbl-dependent part of the folate pathway.

As mentioned earlier, N^5-methyltetrahydrofolate is converted to tetrahydrofolate during the methylation of homocysteine to form methionine. This reaction is the basis for the "methylfolate trap" hypothesis of the etiology of megaloblastosis proposed by Noronha and Silverman in 1961 (53). They propose that Cbl deficiency limits the production of demethylated tetrahydrofolate, "trapping" available folate compounds in methyltetrahydrofolate. This limits the availability of other folate compounds for intracellular metabolism--notably in megaloblastic anemia, it curtails the supply of tetrahydrofolate needed for the conversion of dUMP to dTMP by thymidylate synthetase. The hypothesis is an attractive one: it fits with the theory of pathogenesis of megaloblastosis and explains the response to thymidine, folate, and Cbl in deficiencies of either of the latter two compounds without invoking as-yet-undiscovered Cbl-dependent pathways.

Much confliciting data has been generated in efforts to confirm this hypothesis (43). In vitro studies of the conversion of dUMP to dTMP in bone marrow cells have shown the reaction to be Cbl-dependent (54); methyltransferase activity is reportedly decreased in the liver of Cbl-deficient rats (55); serum levels of N^5-methyltetrahydrofolate was elevated and clearance delayed in a group of patients with Cbl

deficiency (56); and isolated congenital deficiency of methyltransferase has been associated with megaloblastic anemia (57). All this evidence tends to support the proposed "folate trap" mechanism. On the other hand, other measurements of methyltetrahydrofolate utilization in Cbl-deficient patients by studies of clearance and $^{12}CO_2$ generation from $^{14}CH_3$-tetrahydrofolate have shown normal turnover of the methylated compound and have failed to confirm that plasma levels are elevated (58,59). Most difficult to explain are the data provided by children with an inborn error of Cbl metabolism resulting in failure to synthesize either AdoCbl or MeCbl: of the four reported cases of this disorder, only one child had evidence of megaloblastic anemia (60-63). Thus, though the role of Cbl deficiency in megaloblastic anemia is probably mediated by a link to the pathway of folate metabolism, the relationship is not straightforward, and the possibility that Cbl has a completely separate role in some other aspect of folate metabolism has not been ruled out.

Neurologic Disorders -- The hallmark of the neurological disorder associated with Cbl deficiency is "subacute combined degeneration" of the spinal cord--a diffuse, uneven degeneration of myelin sheaths and axon cylinders beginning usually in the posterior columns of the thoracic cord and spreading to the lateral tracts and up and down the cord. The clinical syndrome has its onset in symmetric weakness and paresthesias of the distal extremities and progresses to ataxic and spastic paraplegia, the predominance of ataxia or spasticity depending on the relative involvement of the posterior and lateral tracts. The peripheral signs may be associated with a full range of cerebral manifestations, including irritability, apathy, intellectual deterioration, and psychosis (64).

Little is known of the pathophysiology of subacute combined degeneration. The lack of correlation between neurologic symptoms and megaloblastosis, and the observation that folate can reverse the latter but not the former, have led investigators to believe the Cbl-deficiency-induced demyelination is mediated either through the AdoCbl/methylmalonyl CoA pathway, or through some different mechanism not directly related to either known Cbl-dependent pathway. There are two major pathogenic mechanisms which have been proposed. One suggests that Cbl deficiency blocks the metabolism of methylmalonyl CoA and that accumulation of this compound either inhibits myelin synthesis, or leads to the incorporation of the molecule into abnormal lipids with subsequent synthesis of defective myelin. The other possibility put forward is that Cbl plays a crucial role in the detoxification of cyanide and that subacute combined degeneration is a manifestation of chronic cyanide intoxication secondary to deficiency of the detoxifying mechanism (43).

The first hypothesis has been supported by several *in vitro* studies of fatty acid synthesis; abnormal fatty acids have been shown to be made, in the setting of Cbl deficiency, by rat liver extracts (65), rat glial cells in culture (66), and nerve biopsy slices (67). Once again, however, the clinical manifestations of inborn errors of Cbl metabolism cast doubt on the validity of the theory. Patients with defects in the apoenzyme of methylmalonyl CoA mutase or with defects in the synthesis of AdoCbl present with severe methylmalonic acidemia, but are free of the stigmata of subacute combined degeneration (9).

The cyanide intoxication theory has its roots in the observation that OH-Cbl can combine with cyanide in plasma and can even serve as

an antidote in cyanide poisoning (68,69). The arguments in favor of the failure of this clearance mechanism as the basis of neuropathology in Cbl deficiency rest mainly on the observations of neurologic disorders in cigarette smokers and notably, on observations of the syndrome of tobacco amblyopia, an insidious progression of visual impairment associated with optic atrophy (70,71). Cigarette smokers have an increased CN load, high levels of thiocyanate excretion, and a susceptibility to tobacco amblyopia with mild Cbl deficiency. These observations have been supplemented by other reports of neurologic disorders associated with populations exposed to high levels of dietary cyanide (43). Clinical syndromes of inborn errors of Cbl metabolism serve once again to show that the situation is not so simple. Although the cyanide theory explains why children with isolated errors of AdoCbl metabolism show no signs of subacute combined degeneration, it fails to explain why two of the four children with combined AdoCbl and MeCbl deficiency, but normal serum Cbl levels, did have neurologic symptoms. This argues for the involvement of one of the intracellular pathways of Cbl metabolism in the pathogenesis of this disorder.

Metabolic Ketoacidosis -- Methylmalonic acid excretion is elevated in a large proportion of patients with Cbl deficiency (72), and has been considered a sensitive test which often distinguishes Cbl and folate deficiency (73). The methylmalonic acid excretion in these patients, however, is rarely severe and is not associated with obvious clinical manifestations. By contrast, children with inborn errors of metabolism leading to deficient activity of methylmalonyl CoA mutase, either due to defects in apoenzyme function or coenzyme synthesis, excrete massive quantities of methylmalonic acid and characteristically present with

episodes of life-threatening ketoacidosis, often associated with hypoglycemia, hyperammonemia, and hyperglycinemia (9,74,75).

The pathogenesis of ketoacidosis in these children is not fully understood. The accumulation of methylmalonic acid is easily explained by the block in the conversion of methylmalonyl CoA to succinyl CoA; it also explains why one might expect some elevation of this intermediate to be present in Cbl deficiency. Why this should lead to the other aspects of the syndrome is less obvious, and proposed mechanisms rely on the further role of the mutase reaction in carbohydrate metabolism. Accumulation of methylmalonyl CoA may reduce the availability of coenzyme A and consequently the supply of acetyl CoA for the TCA cycle. Methylmalonic acid is an inhibitor of gluconeogenesis, both by direct inhibition of pyruvate carboxylase and by inhibition of the mitochondrial transport of malate, the precursor of oxaloacetate (76). In addition to causing hypoglycemia, this contributes to the disruption of carbohydrate metabolism; the combined block of the TCA cycle and gluconeogenesis could lead to increased lipolysis and ketoacidosis. The mechanisms of hyperglycinemia and hyperammonemia are unknown (9).

Clinical Manifestations of Transcobalamin II Deficiency

In 1971, Hakami et al. (77) described two siblings who presented at three and five weeks of age with diarrhea, vomiting, and "failure to thrive". Both were found to have severe megaloblastic anemia in the face of normal serum folate and Cbl levels; both responded dramatically to high doses of intramuscular Cbl with complete clinical and hematologic recovery. The infants were found to have a complete absence of TC II. A similar case has been described since (78). In both cases,

pedigree analysis of the families and laboratory studies of their serum suggest a single gene defect with an autosomal recessive mode of inheritance.

In the older of the two index cases, withdrawal of therapy was attempted at one year of age. Megaloblastic anemia recurred in six weeks, and the study was stopped. During this interval, biochemical determinations in urine and plasma revealed no increase in methylmalonic acid excretion , no rise in the blood levels of homocysteine, methionine, or cystathione, and no change in the rate of succinate or propionate oxidation by peripheral leukocytes (79). Despite florid megaloblastic changes, the metabolic variables that reflect the Cbl-dependent pathways remained entirely normal.

Given the crucial role of TC II in the cellular uptake of Cbl, one might expect the presentation of TC II deficiency to resemble most closely that of children with a combined intracellular defect of coenzyme synthesis, who present with methylmalonic acidemia and homocystinuria. In reality, the complete absence of detectable biochemical derangement in these children separates this syndrome both from the classical picture of an acquired Cbl deficiency syndrome and from the "typical" presentation of intracellular defects of Cbl metabolism.

To understand where the syndrome of TC II deficiency fits into the spectrum of Cbl-related metabolic disease, one must analyze the significance of the pleomorphic nature of the manifestations of Cbl-related disorders. Although most patients with acquired Cbl deficiency have methylmalonic acidemia, some do not (43). One might explain this phenomenon by suggesting that in these patients, Cbl deficiency causes megaloblastic anemia and neurologic disorders in susceptible

tissues before it is severe enough to cause measurable changes in blood or urine levels of methylmalonic acid or homocysteine. This argument would explain why acquired Cbl deficiency does not present with the metabolic emergencies typical of intracellular defects leading to absolute deficiencies of Cbl-dependent enzyme function. In this context, the implication of the syndrome associated with TC II deficiency is that here too, the intracellular Cbl deficiency is *not* absolute in all tissues, i.e., that some Cbl is capable of entering cells by a non-TC II-mediated mechanism. This suggestion is supported by the observation of the impressive response of these patients to high doses of Cbl and leads to the hypothesis that human cells possess a mechanism for the uptake and metabolism of free cobalamin. This study was undertaken to investigate that hypothesis.

MATERIALS AND METHODS

Materials

Eagle's Minimal Essential Medium and Earle's Balanced Salt Solution were obtained from Grand Island Biological Co., Grand Island, NY. Fetal calf serum was purchased from Flow Laboratories, Inc., Rockville, MD. HEPES was obtained from Calbiochem, San Diego, CA. Chloroquine was purchased from Sigma Chemical Co.; N-Ethylmaleimide (NEM) was purchased from Eastman. Anti-human TC II antiserum was kindly provided by Dr. R.H. Allen, Division of Hematology, University of Colorado Medical Center. Cyano[^{57}Co]cobalamin (CN-[^{57}Co]Cbl) was purchased from Amersham/Searle.

Cell Culture

Initial experiments were carried out with a representative control line of human diploid skin fibroblasts (line 87). This line has been used in previous work (40,41) to investigate the TC II-mediated uptake of Cbl. Comparative experiments were then performed using a diploid skin fibroblast line (line 181) from a patient with TC II deficiency (77). Cells were grown in Eagle's minimal essential medium supplemented with 1 percent nonessential amino acids, 10 percent fetal calf serum, and 100 µg/ml kanamycin. There is no detectable TC II in fetal calf serum (41). Fetal calf serum contains approximately 300-500 pg/ml Cbl, bound to R-binder. Stock cultures were grown in 32 oz glass bottles which were gassed with 5 percent CO_2/95 percent air, tightly capped, and placed in a 37° incubator. For uptake studies, cells were seeded on 100 x 15 mm Falcon plastic tissue culture plates at 7.5-8.5 x 10^5 cell/dish and placed in a 37° incubator with 5 percent CO_2/95 percent air atmosphere. For Sephadex G150 column experiments, cells were grown at 37° in 150 cm^2 plastic flasks which were gassed and tightly capped. Bottles were seeded at approximately 2 x 10^6 cells/bottle. Cells became confluent on the third day after plating; experiments were performed on day 4.

Uptake of Free CN-[^{57}Co]Cbl

Uptake experiments were carried out in serum-free medium. Preliminary results established that uptake was comparable in the presence or absence of supplementary amino acids. Therefore, subsequent experiments were done in Earle's balanced salt solution (EBSS). With the exception of concentration studies, medium contained CN-[^{57}Co]Cbl at a concentration of 30 pg/ml, a molar concentration equivalent to the Cbl concentration of medium with 10 percent serum saturated with Cbl. The growth medium was aspirated and the cell monolayers washed twice with 5 ml Dulbecco's Ca^{++}- and Mg^{++}-free phosphate-buffered saline (PBS). Six ml of incubation medium or EBSS was added. Initial experiments were carried out at 37° in medium with 10 mM HEPES on a revolving shaker to prevent contribution of an unstirred fluid monolayer. It was determined that shaking had no effect on uptake, and subsequent experiments were carried out without shaking in a humidified CO_2 incubator. After incubations of 30 min to 8 hr, the cells were washed four times with 5 ml PBS, dissolved in 1 ml of 0.2 N NaOH, and transferred to a plastic counting tube. The dish was then rinsed twice using 1 ml of 0.2 N NaOH and rinses combined with the solubilized cells. The solubilized cells were counted in a Packard automatic gamma scintillation counter. An aliquot was assayed for cell protein by the Lowry method (80), and uptake expressed as pg Cbl/mg cell protein.

Studies with metabolic inhibitors were performed by preincubation with inhibitor for a defined interval prior to assay of uptake of CN-[^{57}Co]Cbl. Uptake was then studied in incubation medium containing the same concentration of inhibitor. Concentrations of NaF, cycloheximide, and chloroquine were chosen to match those concentrations

which were used to evaluate the inhibitor profile of TC II-mediated uptake in this system (41). KCN was used in concentrations known to inhibit respiration (81), and NEM concentration was empirically adjusted to find a level that did not prove lethal to fibroblasts. In these experiments, total uptake (binding and internalization) was distinguished from internalization by harvesting with trypsin-EDTA. After incubation of cells at 4°, trypsin-EDTA removed 95-98% of cell-associated ^{57}Co radioactivity (data not shown). Hence, the term "internalization" here applies to ^{57}Co activity associated with cells which is not releasable by trypsin, and "uptake" applies to total cell-associated ^{57}Co activity associated with cells after harvesting with 0.2 N NaOH. For experiments with metabolic inhibitors, cells were harvested by addition of 1 ml of trypsin-EDTA. The dishes were incubated at 37° until the cells were floating. The cell suspension was then transferred to a 15 ml conical centrifuge tube and the dish washed with ice-cold growth medium. The suspension was centrifuged at 1800 g for 3 min at 4°C. The supernatant was removed to a plastic counting tube and counted. The cell pellet was dissolved in 0.2 N NaOH, counted, and cell protein determined.

Intracellular Metabolism of Free Cbl

To determine the fate of CN-[^{57}Co]Cbl taken up by monolayers, cell extracts were prepared and analyzed by Sephadex G150 profiles. The cells were harvested with trypsin and the cell pellet was sonicated for 3 x 15 sec intervals. The extract was centrifuged at 18,000 g for 10 min, then the supernatant was loaded onto a Sephadex G150 (Pharmacia) column and eluted with buffer containing 0.15 M NaCl and 50 mM potassium phosphate, pH 7.4. 1.85 ml fractions were collected and counted for ^{57}Co radioactivity. For column profiles in the presence of chloroquine, the 6 hr uptake was preceded by a one hr preincubation with chloroquine.

RESULTS

Kinetics of Free CN-[^{57}Co]Cbl Uptake

For a preliminary estimate of the magnitude of free CN-[^{57}Co]Cbl uptake, an experiment was performed comparing uptake of CN-[^{57}Co]Cbl at 30 pg/ml in serum-free medium to that in medium containing 10 percent human serum. After a two hour incubation, cells in serum-free medium took up 1.43 pg/mg protein, as compared with an uptake of 6.41 pg/mg protein by monolayers in 10 percent human serum. The latter value is comparable to results of previous experiments with fibroblasts (82).

Fig. 4 shows the time course of uptake in serum-free medium from ten min to eight hrs. Uptake is a biphasic process: the first component is rapid, nonlinear, and complete within less than 30 min; the second phase is linear and does not plateau within eight hrs.

Saturability of Uptake

The uptake of free CN-[^{57}Co]Cbl as a function of concentration is shown in Fig. 5a. To identify non-specific uptake, parallel plates were incubated at each concentration up to 90 pg/ml in the presence of a large excess of unlabelled CN-Cbl. By comparing uptake in the presence and absence of an excess of unlabelled Cbl, the concentration curve can be resolved into two components (Fig. 5b). Uptake in the presence of unlabelled CN-Cbl is linearly proportional to concentration and nonsaturable; this uptake is defined as non-specific. Subtraction of non-specific uptake from total uptake allows derivation of a curve representing an uptake process which is nonlinear and saturable.

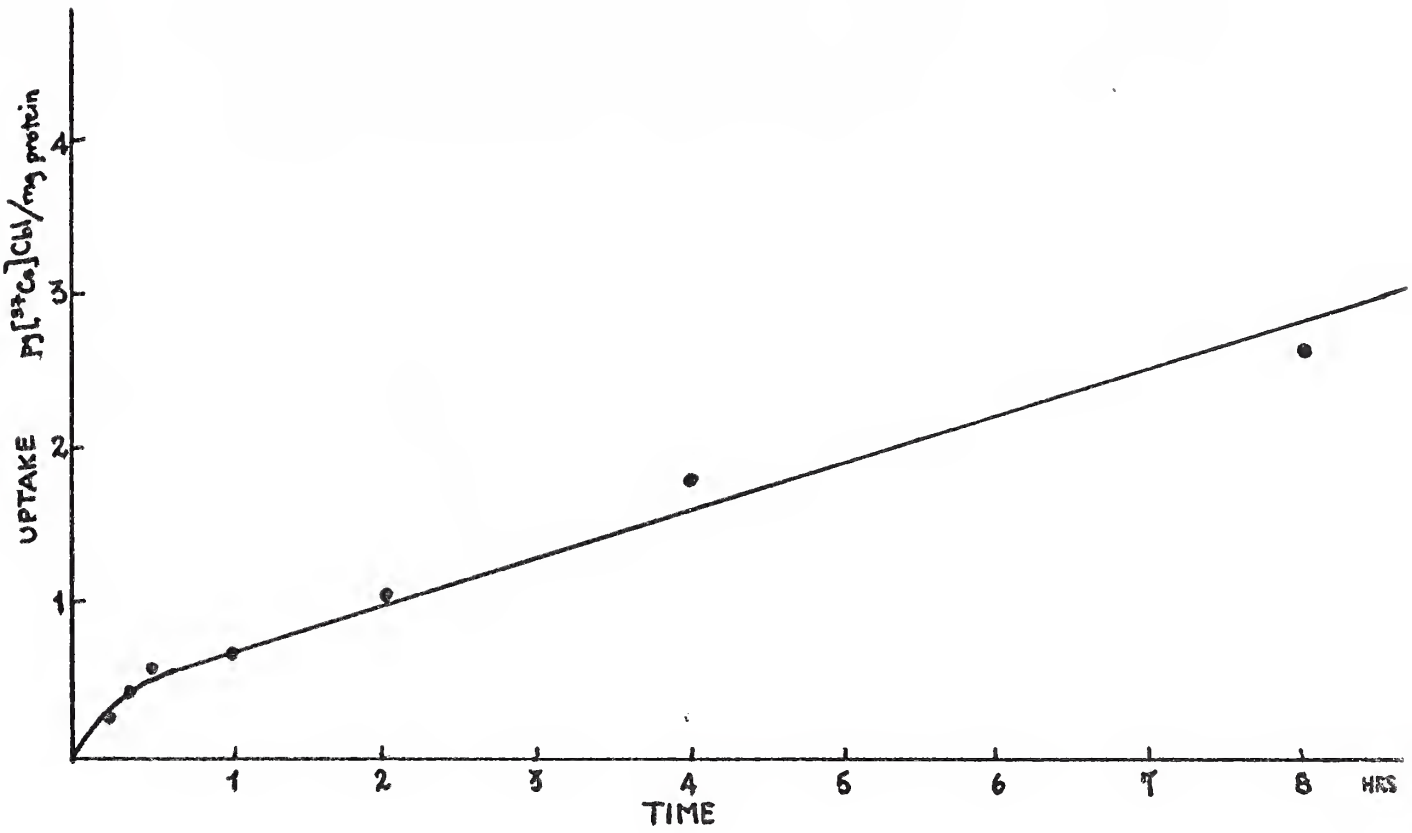


FIGURE 4. Time course of uptake of free Cbl. Cells were incubated at 37° in serum-free medium with 30 pg/ml CN-[^{57}Co]Cbl and solubilized in 0.2 N NaOH. This curve and others to follow show results of a representative experiment; each point is the average of duplicate determinations.

FIGURE 5. Uptake of CN-[^{57}Co]Cbl as a function of concentration. Cells incubated at 37° for two hours. Fig. 5b reproduces part of curve 5a indicated in box. At these concentrations, parallel plates were incubated in the presence of an excess of unlabelled CN-Cbl. Dotted line shows derived saturable component of uptake. See text for details. Closed circles = total uptake. Open circles = nonspecific uptake in the presence of excess unlabelled CN-Cbl.

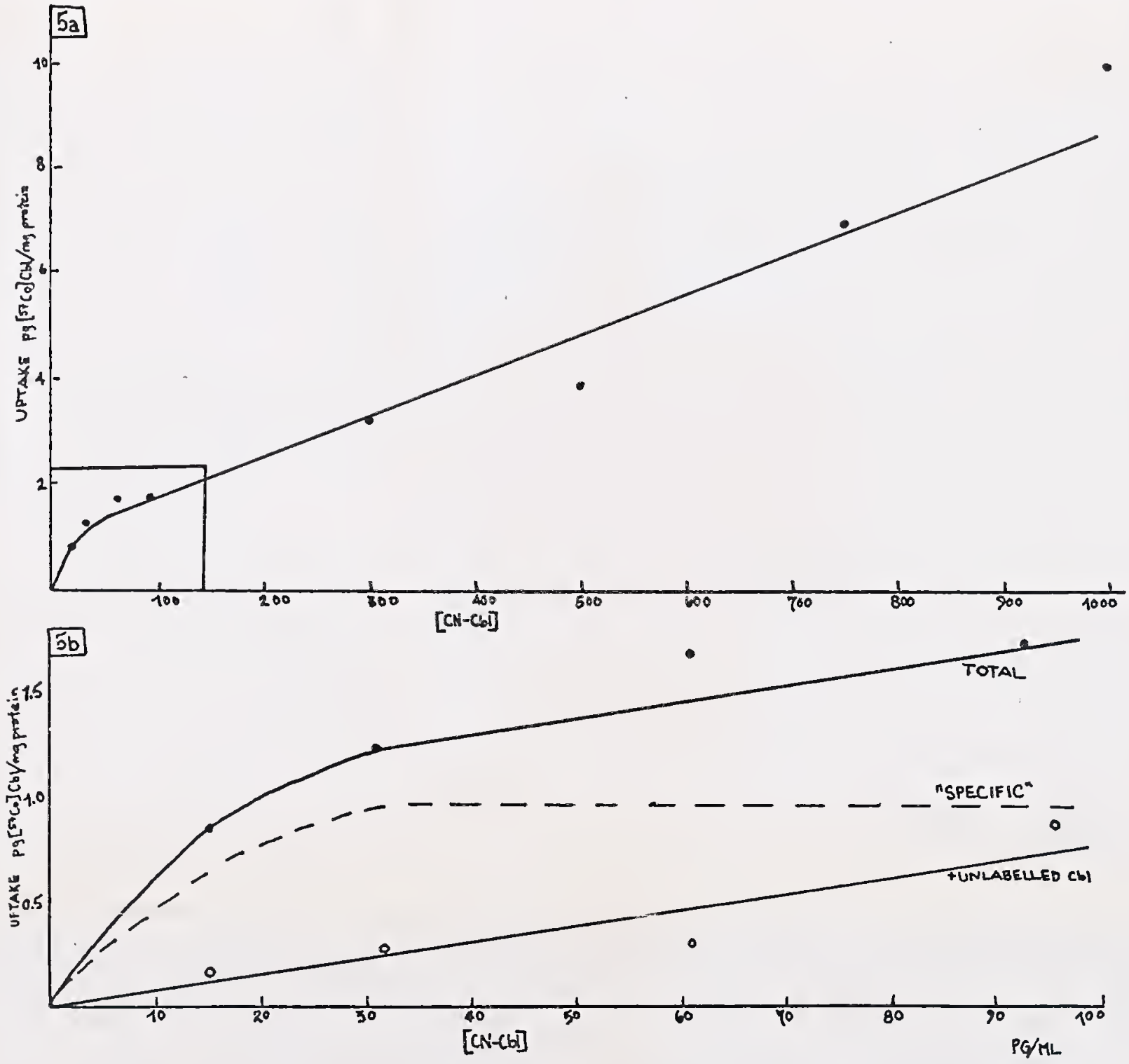

5a
UPTAKE pg [57Co]CbL/mg protein
10
8
6
4
2
100
200
300
400
500
600
700
800
900
1000
[CN-Cbl]
5b
UPTAKE pg[57Co]Cbl/mg protein
1.5
1.0
0.5
TOTAL
"SPECIFIC"
+UNLABELLED Cbl
10
20
30
40
50
60
70
80
90
100
[CN-Cbl]
PG/ML

FIGURE 5

Uptake of OH-Cbl

To determine whether OH-Cbl was equally active in this system, an experiment was performed comparing the effectiveness of unlabelled OH-Cbl and unlabelled CN-Cbl in preventing the uptake of CN-[^{57}Co]Cbl. Fig. 6 reveals that CN-Cbl and OH-Cbl are equally effective in competing for uptake. Subsequent studies of the uptake of OH-[^{57}Co]Cbl as a function of concentration showed uptake comparable to the uptake of CN-[^{57}Co]Cbl (Fig. 7).

Effect of Metabolic Inhibitors on CN-[^{57}Co]Cbl Internalization

Experiments were performed with metabolic inhibitors to determine the metabolic requirements of the internalization of Cbl. Results are summarized in Table 1. Cycloheximide (50 μM), a protein synthesis inhibitor which has been shown previously to inhibit internalization of the TC II-Cbl complex (41), inhibits CN-[^{57}Co]Cbl internalization by 80 percent. NaF (100 μM), a inhibitor of glycolysis, which inhibits TC II-Cbl internalization by 50 percent (41), has no effect on the internalization of free Cbl. KCN in concentrations sufficient to inhibit respiration (10 mM) inhibits free Cbl internalization by only 10 percent. N-ethylmaleimide, which binds sulfhydryl groups, is lethal to cells at 100 μM; at 10 μM, it inhibits internalization of CN-[^{57}Co]Cbl by 76 percent. Chloroquine (50 μM), an inhibitor of lysosomal hydrolase activity, inhibits internalization 25 percent after a 1 hr preincubation and 40 percent after a 2 hr preincubation. Further studies of the effects of chloroquine are presented below.

FIGURE 6. Competition for CN-[^{57}Co]Cbl uptake by excess of unlabelled CN-Cbl and OH-Cbl. Cells incubated at 37° for two hours with 30 pg/ml CN-[^{57}Co]Cbl. Results expressed as percent of control uptake in the absence of unlabelled Cbl.

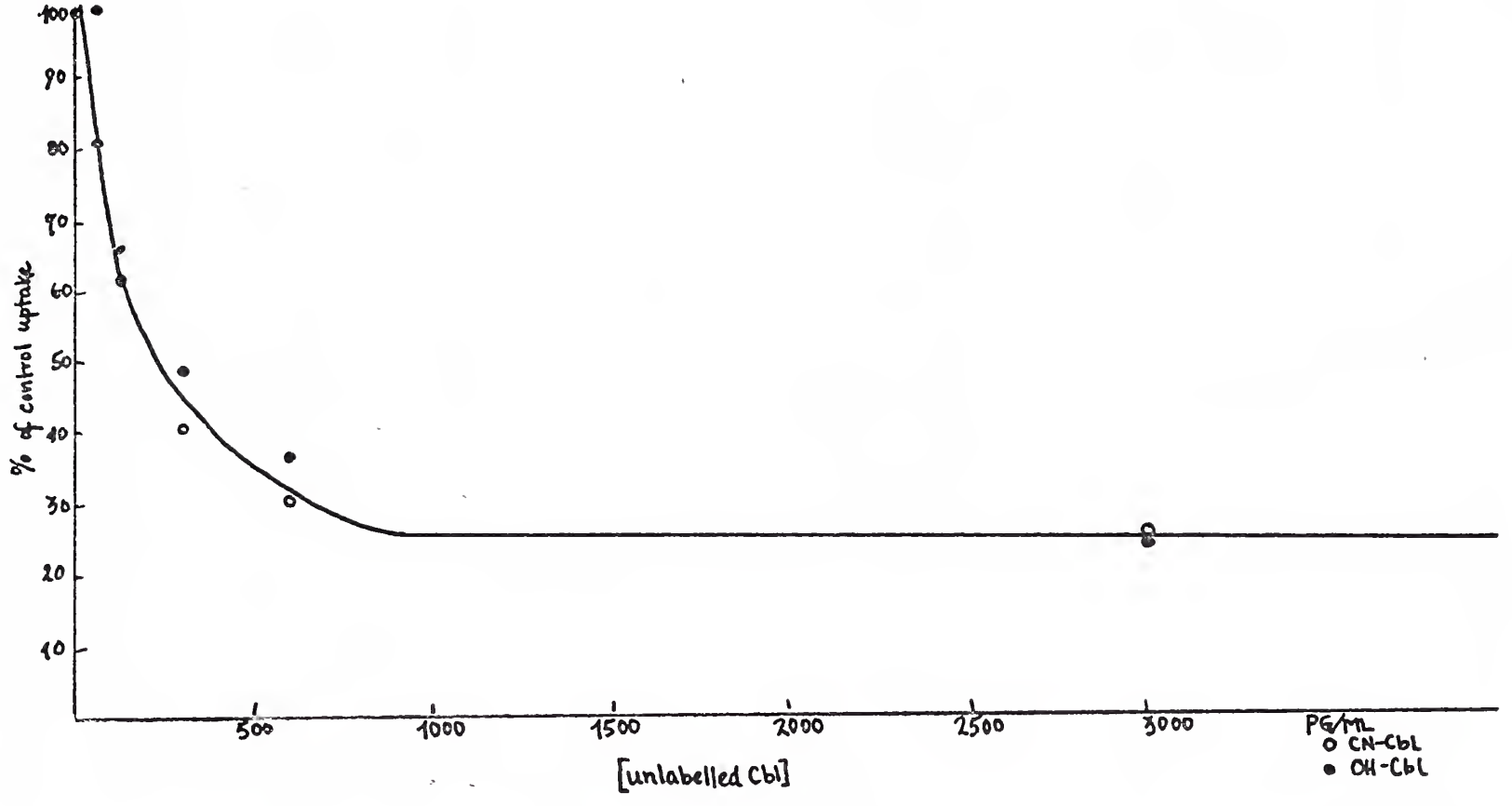

% of control uptake
100
90
80
70
60
50
40
30
20
10
500
1000
1500
2000
2500
3000
[unlabelled Cbl]
PG/ML
o CN-CbL
• OH-CbL

FIGURE 6

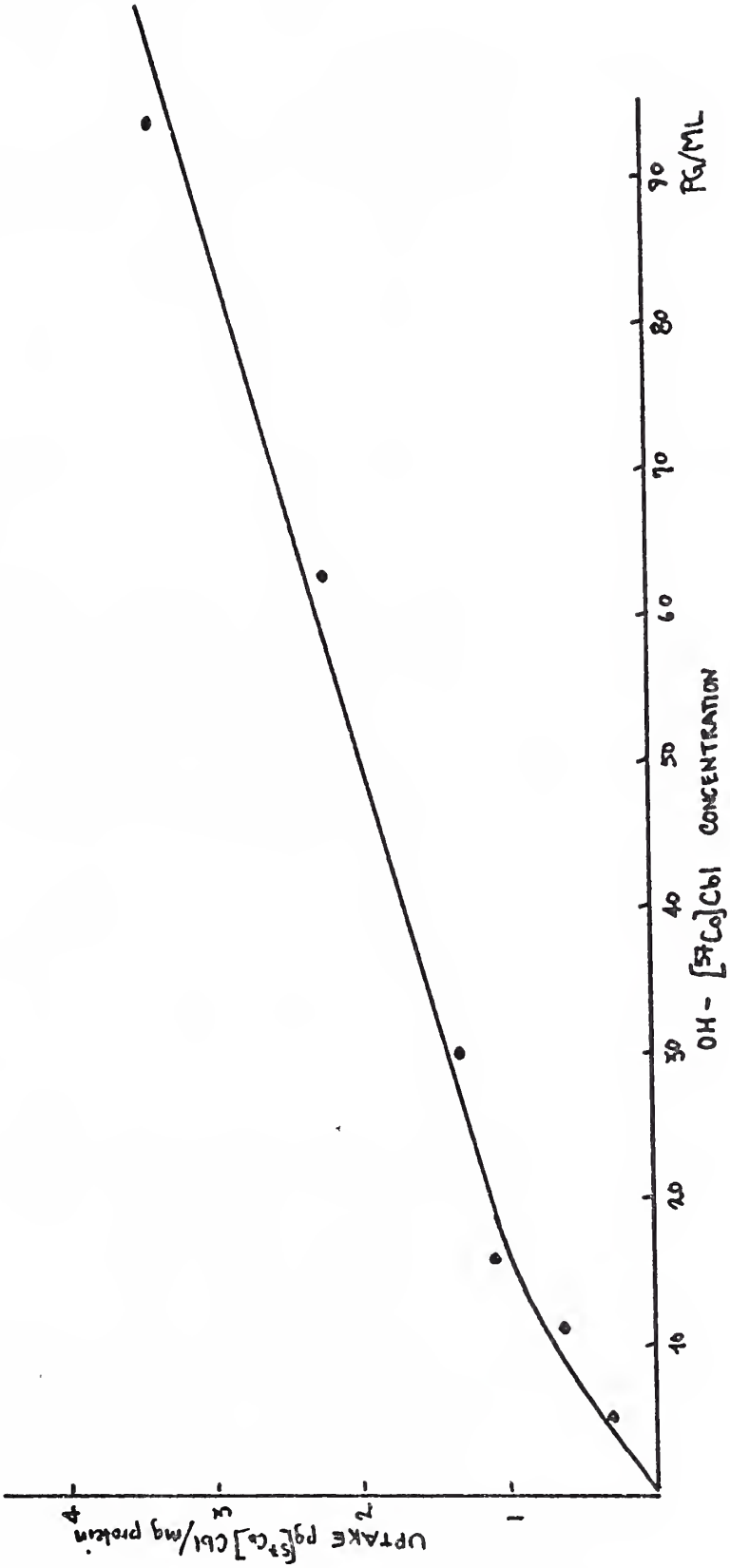


FIGURE 7. Concentration curve for OH-Cbl. Cells incubated at 37° for two hours.

TABLE I

Inhibitor	% Inhibition of Internalization	
	Normal Cells	TC II-deficient Cells
Cycloheximide (50 μM)	80%	44%
NaF (100 μM)	0%	0%
KCN (10 mM)	10%	31%
NEM (10 mM)	76%	41%
Chloroquine (50 μM)	25-40%	0-10%

Table 1: Inhibitor profile for normal and TC II-deficient cells. Cells preincubated in inhibitor for 1 or 2 hrs prior to uptake. Incubation medium for uptake contained 30 pg/ml CN-[^{57}Co]Cbl and inhibitor. After 2 hr uptake, cells were harvested with trypsin-EDTA and inhibition expressed as % inhibition of internalization. Each value is an average of duplicate determinations. A representative experiment is shown.

Intracellular Metabolism of CN-[^{57}Co]Cbl

To confirm that free Cbl taken into cells is metabolized to active coenzymes, a cell extract of monolayers incubated in 30 pg/ml CN-[^{57}Co]Cbl for six hours was loaded on a Sephadex G150 column. The elution profile is shown in Fig. 8. There are three peaks of ^{57}Co activity: the first corresponds to the Cbl-dependent apoenzyme (85), the third represents free Cbl. The second peak co-chromatographs with TC II. To confirm that this peak is, in fact, TC II, a second cell extract was prepared after a six hour incubation. Prior to loading onto Sephadex G150 columns, half was incubated for one hour at 4° with control rabbit serum and the other half with purified rabbit anti-TC II antiserum. The two sera were preincubated with unlabelled CN-Cbl before addition to the sonicate, to saturate endogenous TC II which was found to exist in rabbit serum (data not shown). The results of the two Sephadex G150 profiles are shown in Fig. 9. The three peaks noted above are seen again in the profile of the sonicate incubated with control serum; in the sonicate exposed to anti-TC II antiserum, the second peak moves to the void volume, confirming that this peak is, in fact, TC II. Hence, it appears that fibroblasts make TC II. To determine whether the cells also secrete TC II into the medium, an elution profile of post-incubation medium was obtained. As expected, over 90 percent of ^{57}Co activity was associated with free Cbl; however, there was a measurable peak of TC II-associated radioactivity representing four percent of total ^{57}Co activity.

Studies with TC II-Deficient Fibroblasts

The unexpected discovery that normal fibroblasts make TC II suggested that the saturable component of uptake might, in fact, be TC II-mediated. To investigate this possibility, experiments

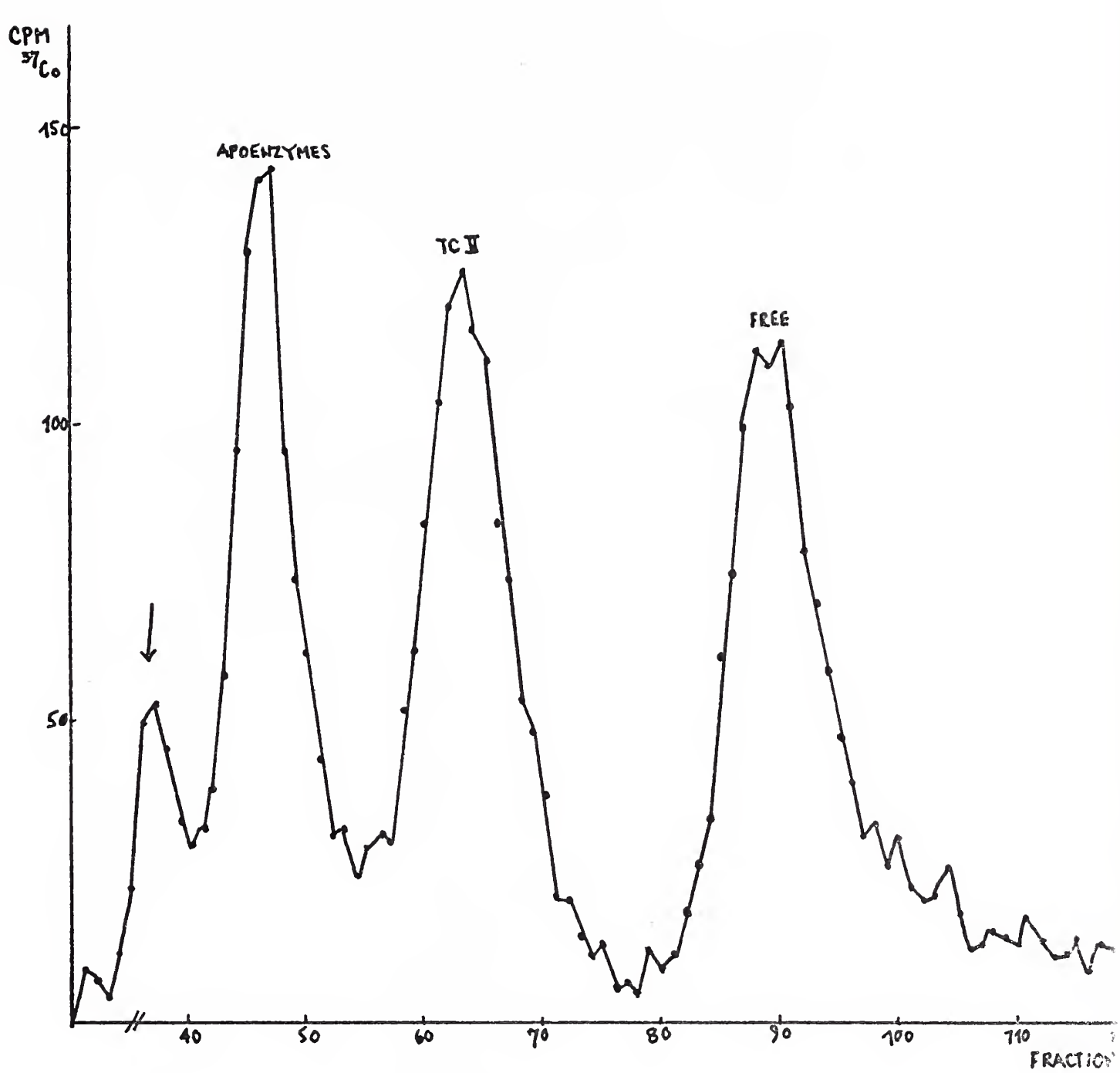


FIGURE 8. Sephadex G150 elution profile of normal fibroblast extract after six hour uptake of CN-[^{57}Co]Cbl. See text for details. Arrow indicates void volume. Recovery was 100 percent.

FIGURE 9. Sephadex G150 elution profile for six hour uptake of CN-[^{57}Co]Cbl by normal fibroblasts. Upper figure shows profile of cell extract incubated for one hour at 4° with control rabbit serum; lower figure shows profile of extract incubated at 4° with anti-TC II antiserum. For details, see text. Recovery was 100 percent.

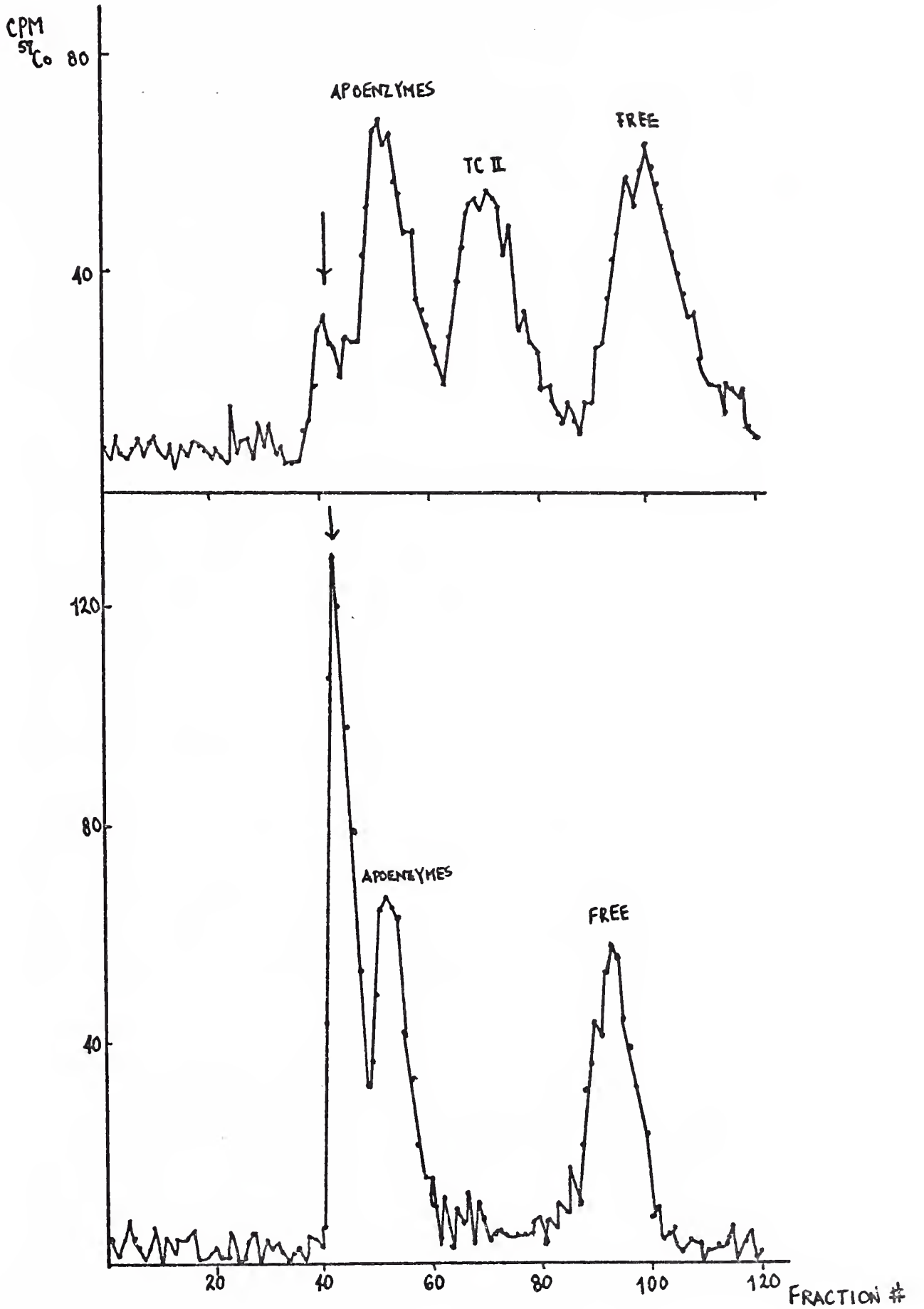

CPM
^{57}Co
80
40
APOENZYMES
TC II
FREE
120
80
40
APOENZYMES
FREE
20
40
60
80
100
120
FRACTION #

FIGURE 9

were repeated with fibroblasts derived from TC II-deficient patients. The uptake of free Cbl by control and TC II-deficient fibroblasts as a function of time and concentration is shown in Fig. 10 and 11. Though at 30 pg/ml the uptake over time is greater for the control cells than the TC II-deficient cells, this difference is not seen at higher concentrations. Once again, the curves for the TC II-deficient cells are biphasic, suggestive of a nonlinear, saturable component of uptake.

A Sephadex G150 elution profile was obtained for the TC II deficient cells. Because the uptake at six hours at 30 pg/ml was so much lower for this cell line, difficulty was encountered in obtaining uptake of sufficient counts to run a column. Consequently, the profile was done after a six hour incubation in 90 pg/ml CN-[^{57}Co]Cbl. A profile was also obtained at 90 pg/ml in control cells. The two elution patterns are compared in Fig. 12. Two characteristics of the TC II deficient cell are remarkable. First, the profile of the mutant cells has no peak corresponding to TC II, thus confirming that these cells are truly deficient in the transport protein. Second, the cells also contain a reduced proportion of free Cbl-associated radioactivity. Almost all of the radioactivity is associated with the single peak corresponding to the Cbl-dependent apoenzymes.

Inhibitor Profile

The inhibitor profile for TC II-deficient cells is compared to that of normal cells in Table 1. Cycloheximide and NEM inhibit CN-[^{57}Co]Cbl internalization significantly, though less than in normal cells. As in controls, NaF does not inhibit internalization of the free vitamin. KCN inhibits internalization of Cbl by 31%, considerably more than in control

FIGURE 10. Comparison of CN-[^{57}Co]Cbl uptake by control and TC II-deficient fibroblasts as a function of time. Closed circles = Control, line 87. Open circles = TC II-deficient cells, line 181.

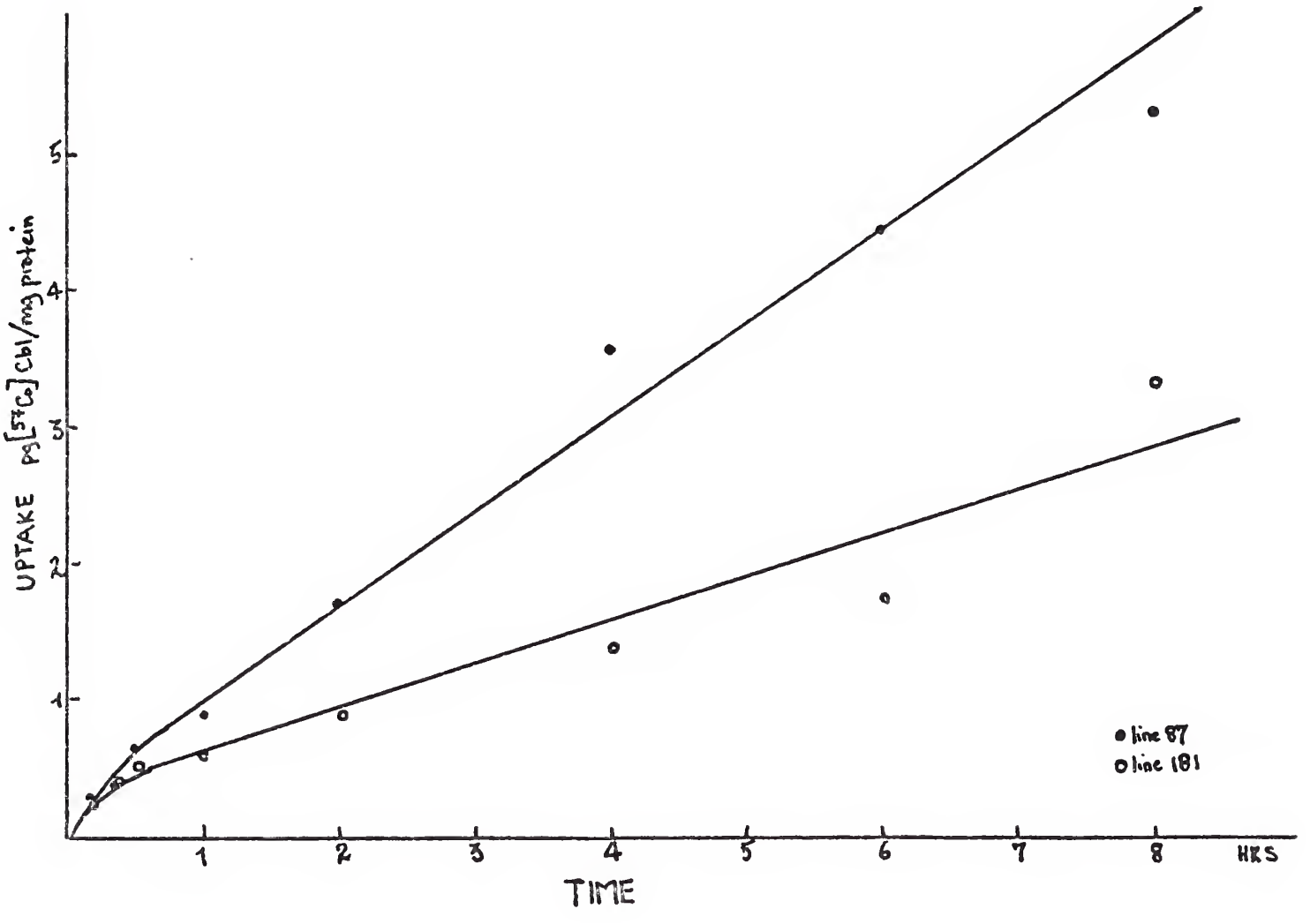

UPTAKE pg[57Co] Cbl/mg protein
1
2
3
4
5
1
2
3
4
5
6
7
8
HRS
TIME
● line 87
○ line 181

FIGURE 10

FIGURE 11. Comparison of CN-[^{57}Co]Cbl uptake by control and TC II-deficient fibroblasts as a function of concentration. Closed circles = control, line 87. Open circles = TC II deficient cells, line 181.

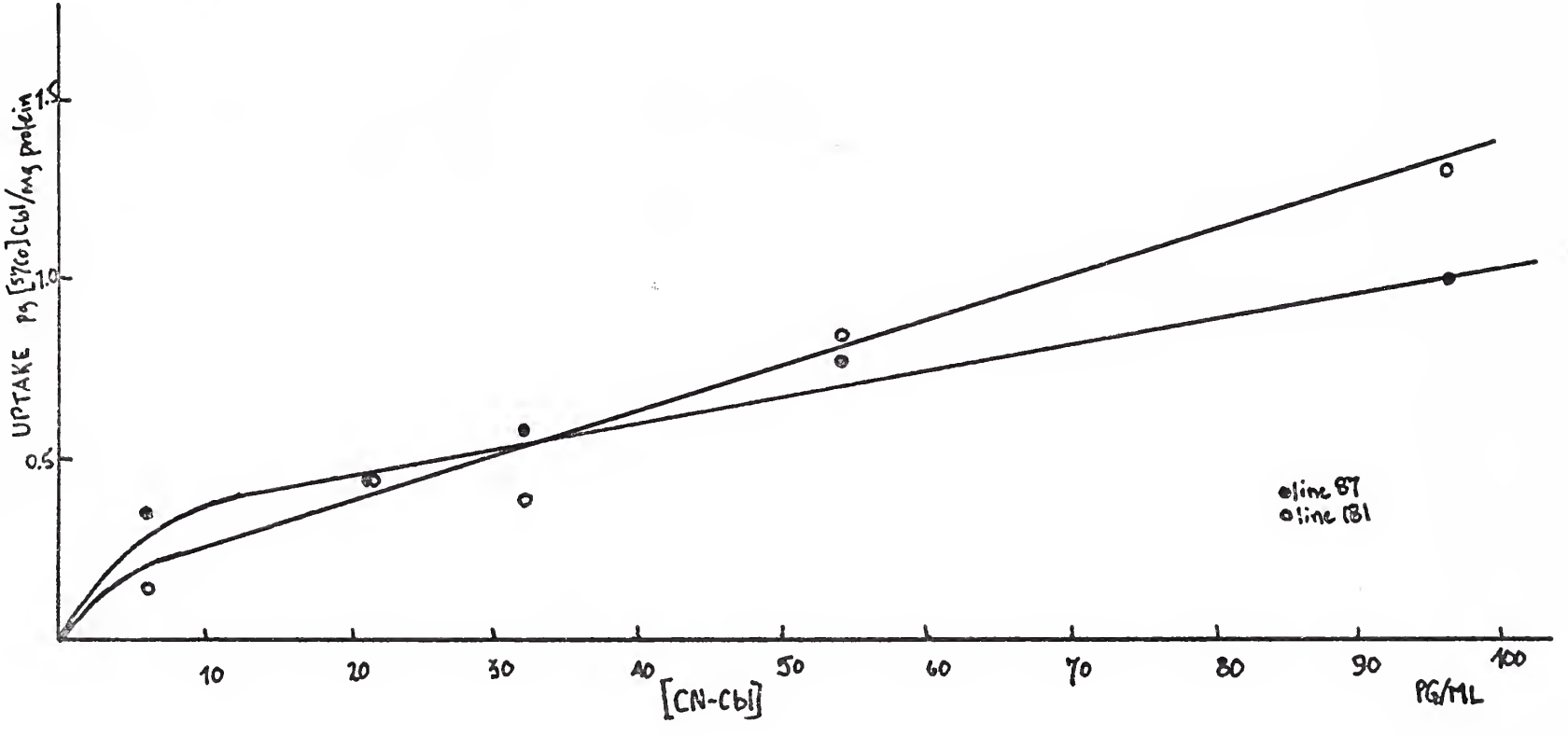

UPTAKE pg [57Co]Cbl/mg protein
0.5
1.0
1.5
10
20
30
40
50
60
70
80
90
100
[CN-Cbl]
PG/ML
●line 87
○line 181

FIGURE 11

FIGURE 12. Comparison of Sephadex G150 profile of cell extracts of control (87) and TC II-deficient (181) fibroblasts after 6 hr incubation in 90 pg/ml CN-[^{57}Co]Cbl. Free Cbl-associated ^{57}Co activity represents 25 percent of non-TC II associated activity in control cells, as compared with 10 percent of ^{57}Co activity in mutant cells. Arrow indicates void volume. Recovery was 100 percent.

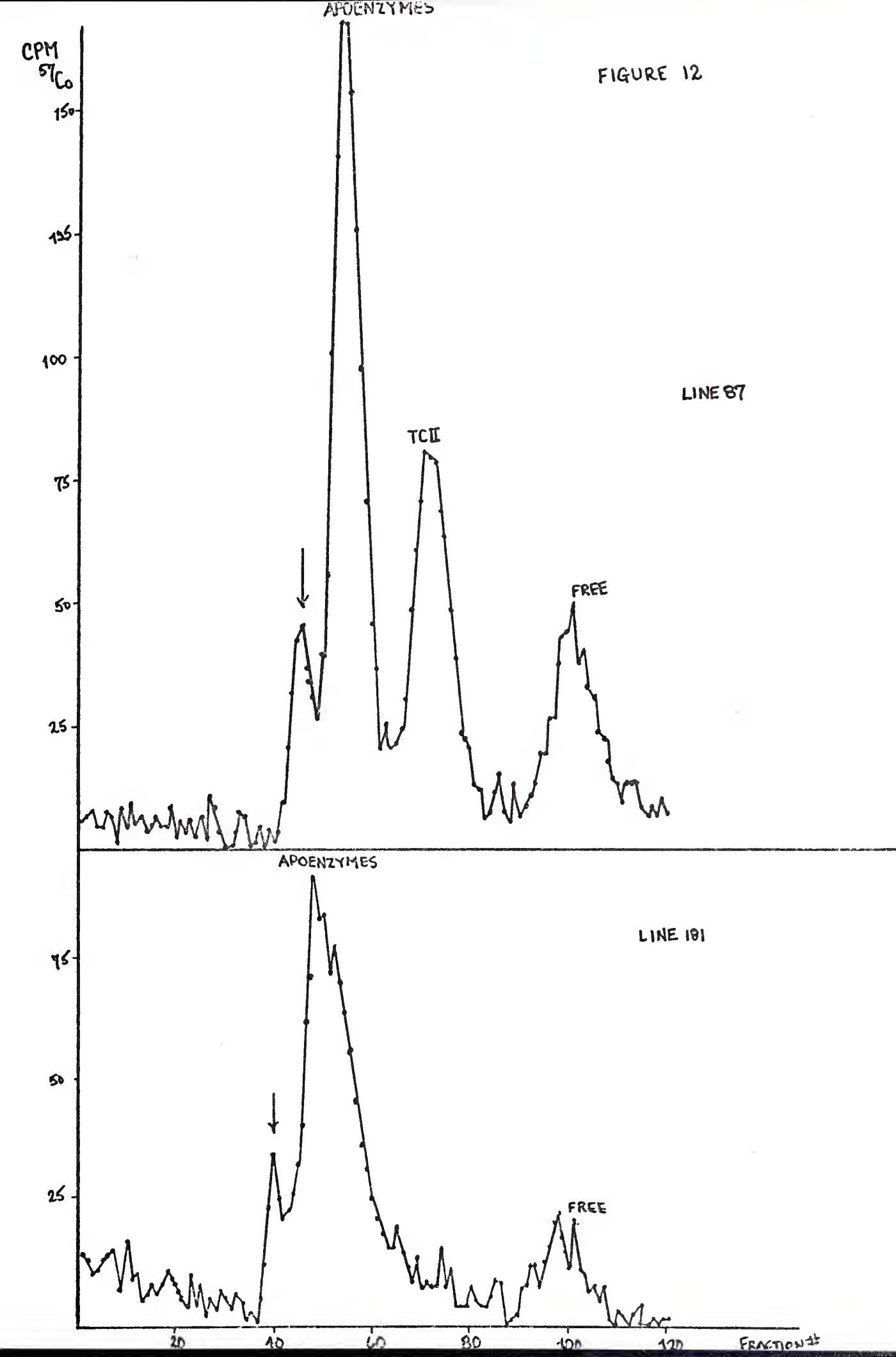

APOENZYMES
CPM
57Co
150
125
100
75
50
25
TCII
FREE
LINE 87
APOENZYMES
75
50
25
FREE
LINE 191
20
40
60
80
100
120
FRACTION #

FIGURE 12

cells. This was noted to be associated with a 15 percent decrease in cell protein in those dishes, and may indicate that for some reason, KCN is especially toxic to these cells.

Effect of Chloroquine on Uptake of Free Cbl

To investigate the role of lysosomes in the uptake process and to further analyze the inhibition of free Cbl uptake by chloroquine a Sephadex G150 profile was obtained of a cell extract of fibroblasts (line 87) which had been preincubated for one hour in chloroquine and then incubated for six hours in uptake medium with 90 pg/ml CN-[^{57}Co]Cbl and 50 μM chloroquine. The profile is shown in Fig. 13. Uptake in the presence of chloroquine leads to peaks of free Cbl and apoenzyme-associated ^{57}Co activity which are much reduced over control conditions (compare to Fig. 12). The TC II-associated activity is greatly increased.

Internalization of Cbl by TC II-deficient cells in the presence of chloroquine (Fig 14) is essentially unchanged. There is still a single major peak of radioactivity associated with apoenzymes and little free CN-[^{57}Co]Cbl.

FIGURES 13 and 14. Sephadex G150 uptake profile of cell extract of control cells (line 87) and TC II-deficient cells (line 181) in presence of chloroquine. Cells were preincubated for one hour in 50 μM chloroquine, followed by a 6 hr incubation in 90 pg/ml CN-[^{57}Co]Cbl and 50 μM chloroquine. See text for details. Free Cbl peak represents 13 percent of ^{57}Co activity in mutant cells, and 10 percent of ^{57}Co activity in control cells. TC II-associated activity in control cells is increased from 27 percent under control conditions (Fig. 12) to 45 percent of ^{57}Co activity; apoenzyme-associated activity is decreased from 36 percent to 22 percent.

FIGURE 13

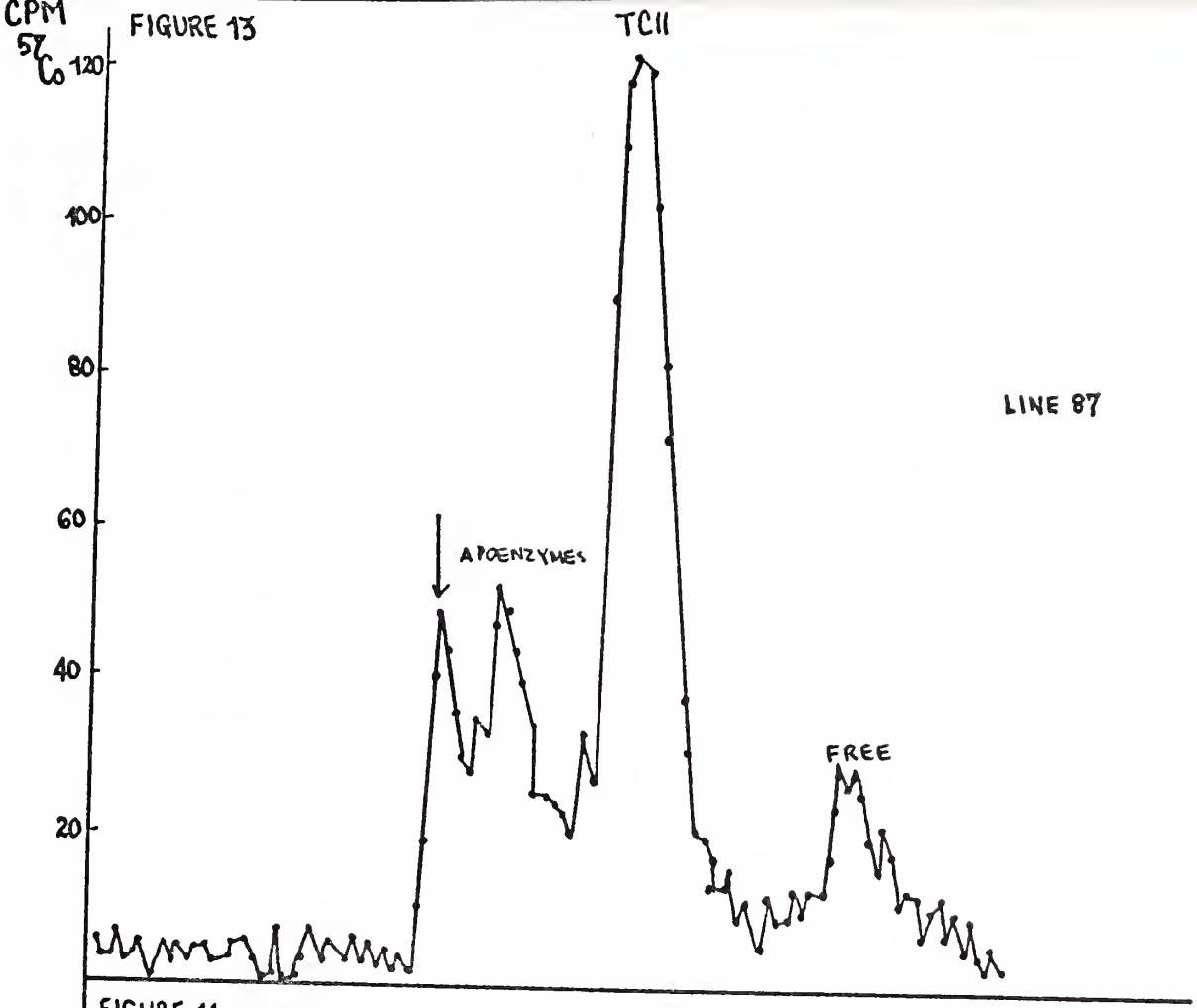
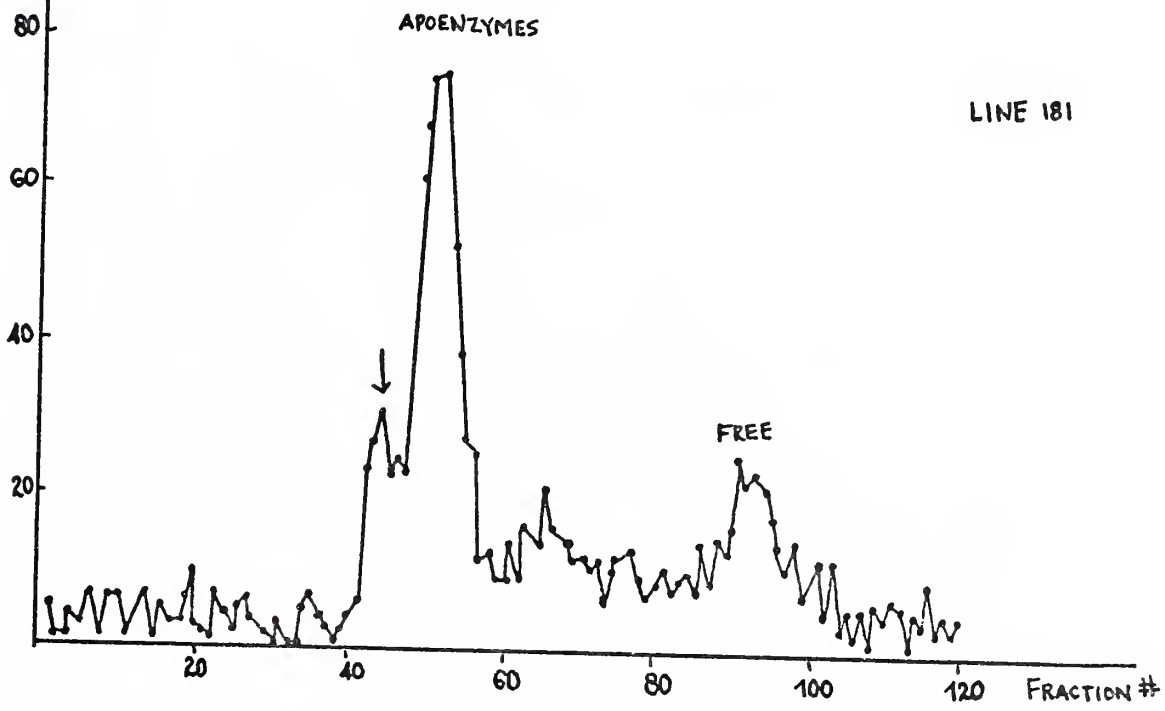


FIGURE 14



DISCUSSION

The experiments described above lead to two major conclusions: 1) that normal human diploid fibroblasts in tissue culture synthesize TC II and secrete it into culture medium, a property not observed in cultured mutant fibroblasts derived from patients with serum deficiency of that protein, and 2) that normal and TC II-deficient cells share a mechanism for the uptake of free Cbl which is independent of TC II.

Evidence for the synthesis and secretion of TC II by fibroblasts in culture lies in the observation of a CN-[^{57}Co]Cbl binding capacity which co-chromatographs with TC II on Sephadex G150, which reacts with purified anti-human TC II antiserum, and which accumulates in incubation medium over time. The observation that TC II-synthetic capability is expressed by fibroblasts in culture allows the further characterization of the defect which is expressed as complete absence of TC II in serum. Fibroblasts cultured from TC II-deficient patients lack Cbl-binding capacity in the TC II region of the Sephadex G150 profile; this suggests that these cells either do not synthesize TC II or that they synthesize an abnormal protein which is unable to bind Cbl.

Although the synthesis of TC II by human cells in culture has not been described previously, synthesis of the transport protein has been reported in cultured rat parenchymal cells (84) and mouse fibroblasts (85). The *in vivo* significance of the *in vitro* synthesis of a serum transport protein by cell lines serially propagated in culture is difficult to evaluate. The production of TC II has also been observed in isolated, perfused dog heart, spleen, kidney, and liver (86). The latter two organs have been considered sites of synthesis and secretion of TC II (28), but the heart and spleen would be unusual secretory sources

of plasma transport proteins; their production of TC II argues for widespread endogenous synthesis of TC II in mammalian tissues.

If these observations in vitro are considered to reflect widespread synthesis of TC II in vivo, one might then speculate as to the functional significance of that synthesis. Hall and Rappazzo (28) have reported that the capacity for uptake of free Cbl is enhanced in those organs which produce TC II. However, one cannot suggest that endogenous TC II functions to facilitate uptake of free Cbl, since, with the possible exception of the liver, peripheral tissues are exposed only to Cbl which is already bound to transport proteins. Alternatively, peripheral production of TC II may be an evolutionary vestige with no physiologic significance, although the possibility that TC II plays an as-yet-unknown intracellular function has not been excluded.

These experiments have also identified a TC II-independent uptake process for Cbl common to both control and TC II-deficient cells. This process is biphasic, with a saturable, specific component which is saturated at physiologic concentration and which reaches asteady state within thirty minutes. Uptake is inhibited by inhibitors of protein synthesis and by disulfide bond reductants; it is relatively unaffected by inhibitors of glycolysis and respiration. These characteristics suggest a specific, receptor-mediated process which is energy-independent; it is most consistent with a process of facilitated diffusion.

A free Cbl-specific receptor has been described in bacterial systems (87,88); Fiedler-Nagy et al. have also reported specific binding of free Cbl to preparations of rat liver membranes (89). The latter is the only other evidence to date of a specific uptake mechanism for free Cbl in higher animals. The results reported in rat liver

membranes are remarkable for the observation of binding of free Cbl of a magnitude similar to that mediated by TC II. The Fiedler-Nagy experiments measured binding, rather than total uptake. However, if the first component of uptake is considered to represent binding, as was proposed in early studies of TC II-Cbl uptake (36,37), the observation by Fiedler-Nagy of approximately equal binding of free Cbl and TC II-Cbl differs from previous observations of facilitation of Cbl uptake (binding and internalization) by TC II (34,35), as well as from the results obtained in the present study. The reason for this discrepancy is unclear, although the possibility exists that this unusual avidity for free Cbl is a property unique to liver parenchyma. Such an avidity might explain the clinical manifestations of TC II deficiency and provide insight into the physiologic significance of the saturable uptake process for free Cbl. The uptake of free Cbl when present in physiologic concentrations is inadequate in stem cells of rapidly proliferating tissue to prevent megaloblastic transformation, mucosal ulcerations, and intestinal malabsorption. However, one may infer that it is adequate in liver parenchyma to prevent methylmalonic acidemia and homocystinuria, since these abnormalities do not develop. Whether this suggests that the liver's demand for Cbl is less, or its avidity for the vitamin more than that of stem cells, is unclear.

At the pharmacologic doses which successfully treat TC II deficiency, which leads to serum Cbl concentrations of 2000-5000 pg/ml, uptake is primarily nonspecific. Whether uptake of free Cbl at these concentrations occurs by simple diffusion, bulk phase pinocytosis, or some other non-specific process remains unclear.

Comparison of the Sephadex G150 profiles of normal and TC II deficient cells exposed to CN-[^{57}Co]Cbl provides further insight into

the mechanism of uptake of free Cbl. The absence of significant free Cbl-associated radioactivity in TC II-deficient cells suggests that the presence of intracellular free Cbl is a characteristic only of TC II-mediated uptake. These observations could be explained by the role of lysosomes in the metabolism of the TC II-Cbl complex as follows: as discussed in the introduction, the TC II-Cbl complex is thought to be taken up intact by adsorptive endocytosis and digested within lysosomes; this results in the release of free Cbl and its subsequent transport out of the lysosome into the cytoplasm where it can be metabolized to its active forms. Thus, it seems possible that the free Cbl-associated ^{57}Co activity in normal fibroblasts may represent Cbl still residing within lysosomes where it is unavailable for immediate conversion to coenzymes. It then follows that the association of all ^{57}Co activity in mutant cells with Cbl-dependent enzymes implies that the internalization of Cbl by these cells is followed by synthesis of coenzymes and association with apoenzymes with sufficient rapidity to prevent accumulation of free Cbl; i.e., that free Cbl is not taken up into lysosomes, but enters the cytoplasm directly. This hypothesis is supported by the observation that chloroquine, which leads to the accumulation of TC II-Cbl within lysosomes (41), abolishes most of the free Cbl-associated radioactivity in normal cells. Thus it appears that the uptake of free Cbl by fibroblasts cccurs by receptor-mediated facilitated diffusion which leads to direct entry of the vitamin to the cytoplasm.

If this uptake process is considered to occur in vivo, one might again speculate as to the physiologic significance of the observations which have been made. Since the TC II-mediated uptake process is more

efficient and is certainly the crucial means of uptake in those cells which have large Cbl requirements, it is not obvious what evolutionary pressures might exist for maintenance of a mechanism for the uptake of free Cbl. There is nothing to exclude the possibility that the mechanism observed in these experiments is merely a remnant of an evolutionary period preceding the development of more efficient uptake processes. However, there is a place for transport systems for free Cbl in mammalian tissues. Though the plasma transport and cellular uptake of Cbl is mediated by TC II, intracellular metabolism of the TC II-Cbl complex requires the movement of Cbl out of lysosomes and into mitochondria. This suggests a scheme whereby the free Cbl uptake system described here might have a role in normal Cbl metabolism. In the course of endocytosis of the TC II-Cbl complex, a portion of the plasma membrane becomes first incorporated into a phagosome, and subsequently becomes part of the lysosomal membrane. In this process the plasma membrane becomes everted. If that membrane fragment contained a receptor for free Cbl capable of mediating facilitated diffusion of Cbl <u>into</u> the cell, it might then neatly fulfill a function as a mediator of free Cbl passage <u>out</u> of the lysosome (Fig. 15). Certainly this proposed mechanism at present lies in the realm of pure speculation; it does, however, describe a cellular economy which is more appealing than the relegation of the uptake process to the category of evolutionary castoffs which have lost their function.

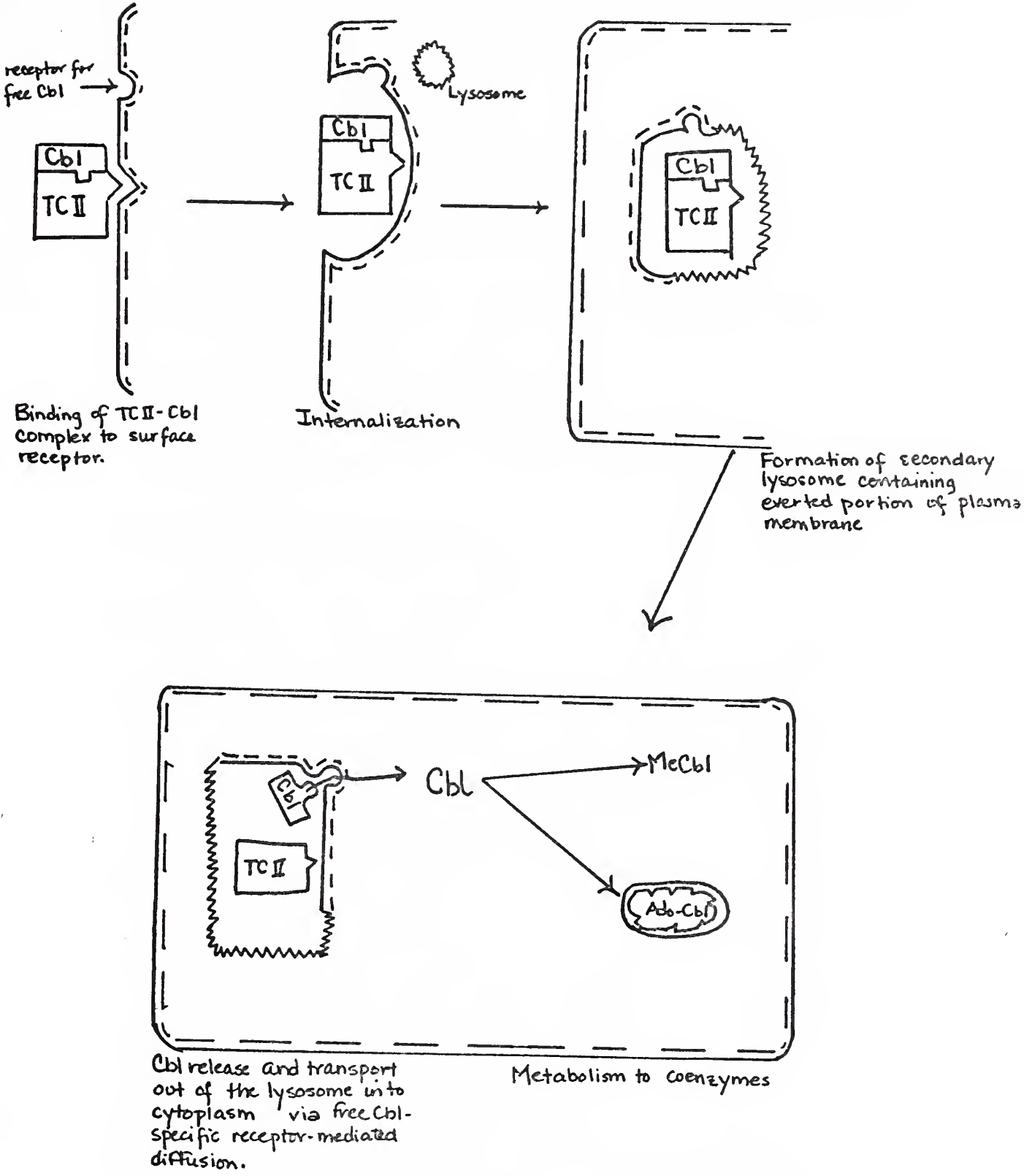


FIGURE 15. Proposed mechanism of role of free-Cbl uptake process in intracellular Cbl metabolism. See text for details.

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